AD_____

Award Number: DAMD17-02-1-0089

TITLE: An MR Contrast Agent for Intra-Prostatic Imaging of Prostatic Cancer

PRINCIPAL INVESTIGATOR: Lee Josephson, Ph.D.

CONTRACTING ORGANIZATION: Massachusetts General Hospital
Boston, MA 02114

REPORT DATE: January 2004

TYPE OF REPORT: Annual

PREPARED FOR: U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

DISTRIBUTION STATEMENT: Approved for Public Release;
Distribution Unlimited

REPORT DOCUMENTATION PAGE

Form Approved
OMB No. 074-0188

1. AGENCY USE ONLY (Leave blank)	2. REPORT DATE	3. REPORT TYPE AND DATES COVERED
	January 2004	Annual (1 Jan 2003 - 31 Dec 2003)

4. TITLE AND SUBTITLE

An MR Contrast Agent for Intra-Prostatic Imaging of Prostatic Cancer

5. FUNDING NUMBERS

DAMD17-02-1-0089

6. AUTHOR(S)

Lee Josephson, Ph.D.

7. PERFORMING ORGANIZATION NAME(S) AND ADDRESS(ES)

Massachusetts General Hospital
Boston, MA 02114

E-Mail: josephso@helix.mgh.harvard.edu

8. PERFORMING ORGANIZATION REPORT NUMBER

9. SPONSORING / MONITORING AGENCY NAME(S) AND ADDRESS(ES)

U.S. Army Medical Research and Materiel Command
Fort Detrick, Maryland 21702-5012

10. SPONSORING / MONITORING AGENCY REPORT NUMBER

11. SUPPLEMENTARY NOTES

12a. DISTRIBUTION / AVAILABILITY STATEMENT

Approved for Public Release; Distribution Unlimited

12b. DISTRIBUTION CODE

13. ABSTRACT (Maximum 200 Words)

The goal of the current research is the development of a magnetic nanoparticle based MR contrast agent that binds the gastrin releasing peptide receptor, a molecular marker associated with neoplasia. In the first year of this proposed work a series of bombesin derivatives have been synthesized and their binding to the GRP receptor quantitated by displacement of radioiodinated bombesin. Positively charged derivatives have lower LC_{50}s, suggesting that the environment around the binding pocket is negatively charged and hydrophilic. Selected peptides have been conjugated to magnetic nanoparticles with various numbers of peptides per nanoparticle. A non-isotopic, enzyme immunoassay method, based on the ability to quantify fluorescein, has been developed to replace the assay using receptor binding and displacement of radioiodinated peptides.

14. SUBJECT TERMS

Prostate Cancer

15. NUMBER OF PAGES

30

16. PRICE CODE

17. SECURITY CLASSIFICATION OF REPORT	18. SECURITY CLASSIFICATION OF THIS PAGE	19. SECURITY CLASSIFICATION OF ABSTRACT	20. LIMITATION OF ABSTRACT
Unclassified	Unclassified	Unclassified	Unlimited

Table of Contents

Introduction

An MR contrast agent targeted to the GRP receptor will be a novel pharmaceutical capable of non-invasively, and at high spatial resolution, characterizing healthy and patholological regions within the prostate. The agent will be an iron oxide nanoparticle conjugated to a bombesin-like peptide. MR imaging of prostate, combined with information on a molecular marker of the neoplastic state, will provide new useful information about intra-prostatic receptor distribution, and the intra-prostatic distribution of the neoplastic state. The goal of the research is to develop a magnetic nanoparticle MR contrast targeted to the gastrin releasing peptide receptor (GRP receptor) that will be used to image the intra-prostatic distribution of this key molecular marker.

Results and Discussion (Body)

The second year of our work plan was carried out. We synthesized additional bombesin-like peptides and conjugated them to CLIO nanoparticles. We determined nanoparticle uptake by prostate cancer cells was a function of valency (number of peptides attached per nanoparticle) and type of charge used for linker amino acids. We characterized the growth promoting properties of the peptide-nanoparticle conjugates and found they lacked the growth promoting properties of peptides. Previously, in year 1, we determined the interaction of bombesin-like peptides with the gastrin releasing peptide receptor using a radioreceptor method. In year two we focused on the behavior of peptide-nanoparticle conjugates and used a non-isotopic immunoassay method (FITC-hapten immunoassay) to assess their interaction with gastrin releasing peptide receptor bearing PC-3 cells.

I. Synthesis and characterization of bombesin-like peptides and peptide-nanoparticle conjugates

Bombesin-like peptides were synthesized as given in table 1.

Table 1: Sequence of bombesin-like peptides synthesized in Year 2

Designation	Peptide Sequence
S	FITC-bACsssGQRLGNQTAVGHLM
R	FITC-bACrrrGQRLGNQTAVGHLM
D	FITC-bACdddGQRLGNQTAVGHLM

We then prepared peptide-nanoparticles conjugates. where we varied the valency or number of peptides/nanoparticle as shown in Table 2. The charge on the nanoparticles was varied as well, where S=serine = neutral, R=arginine=positive, D=aspartate=negative.

Table 2: Summary of peptide-nanoparticles made (year 2)

Peptide	valency	nanoparticle
S	5.1	S5-CLIO
S	9.1	S9-CLIO
S	15.3	S15-CLIO
R	7.1	R7-CLIO
R	23	R23-CLIO
R	34	R34-CLIO
D	4.8	D5-CLIO

II. Determination of peptide and peptide-nanoparticle interaction with the GRPR

receptor (Gastrin releasing peptide receptor).

FITC-Hapten Immunoassay method to determine interactions with GRPR on cells

We completed our development of an FITC-hapten immunoassay methodology for use in characterizing the interaction of peptide-nanoparticles with prostate cancer cells without the use of radioactivity. A submitted manuscript is supplied as an addendum. Figure 1 compares the FITC-hapten immunoassay method with the radioactive receptor method for the binding bombesin to the gastrin releasing peptide receptor expressed on prostate cancer PC-3 cells.

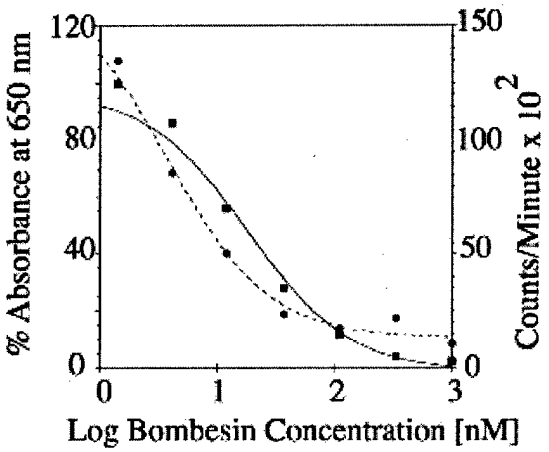


Figure 1: Binding to PC-3 cells determined by FITC-hapten immunoassay and radioreceptor assay. ■, % absorbance; ●, radioactivity.

Additional data with the assay are summarized in Table 3. As expected there was no binding to HT-29 cells, which lack the gastrin releasing peptide receptor. As can be seen from Table 3, the FITC-hapten immunoassy gave similar numbers of binding sites per cell, and a similar IC50, as the radio-receptor assay.

Table 3: FITC-hapten and radioreceptor assays for bombesin binding

Cells	Assay	IC_{50} (nm)	Sites/Cell
PC-3	FITC-hapten	8.7	14,000
PC-3	Radio receptor	6.3	13,000
HT-29	FITC-hapten		<1000

Interaction of peptide-nanoparticles with the GRP receptor

We have examined the effects of attaching different numbers of peptides to the CLIO nanoparticle as shown in Table 4. Data are for the uptake of nanoparticles by PC-3 cells measured by the FITC-hapten immunoassay with increasing concentrations of nanoparticle. Cell associated nanoparticle was determined by the FITC-hapten immunoassay. Data were analyzed according to a 4 parameter equation and EC50 values are provided. Nanoparticles with a spacer of arginine between the GRP receptor binding amino acids and the CLIO nanoparticle appear to the optimal choice for the design of intra-prostatic MR contrast agent.

Table 4: Uptake of peptide-nanoparticles by PC-3 cells.

Nanoparticle	EC50 (uM Fe)
S5-CLIO (10)	>100
S9-CLIO (30)	>100
S15-CLIO (70)	Not obtained
R7-CLIO (10)	10.8
R23-CLIO (30)	1.38
R34-CLIO (34)	0.16
D5-CLIO	>100

Effect of peptide nanoparticles on cell proliferation

We next examined the effects of our peptide-nanoparticle conjugates on the proliferation of PC-3 cells, because the stimulation of cell growth is an undesirable property for diagnostic agents. We examined total cell enzyme activity using an MTT assay, counted cell number, and measured cell DNA synthesis using a BUDR uptake assay (Figures 2-4). While the bombesin peptide stimulated cell growth, peptide-nanoparticle conjugates were not growth stimulatory.

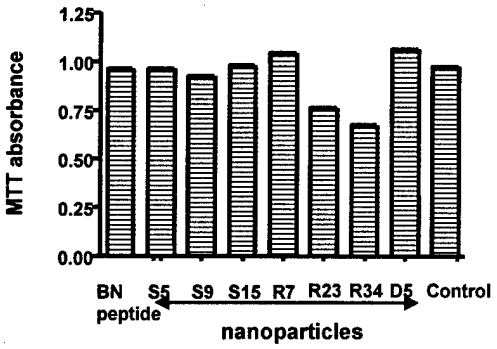


Figure 2: Effect of peptides on peptide-nanoparticles on cells (MTT assay)

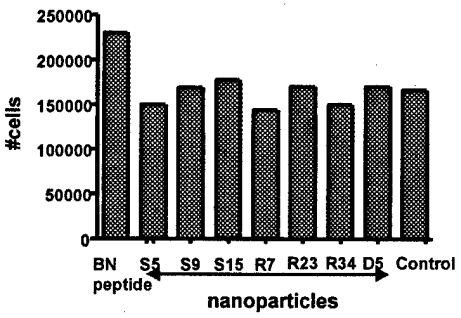


Figure 3: Effect of peptides on peptide-nanoparticles on cells (cell number)

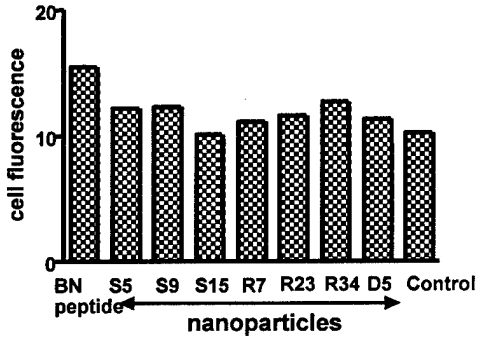


Figure 4: Effect of peptides on peptide-nanoparticles on cells (BUDR uptake assay)

Key Research accomplishments

- We completed the development of the FITC-Hapten immunoassay for use with uptake studies of bombesin-like peptides and peptide-nanoparticle conjugates. See manuscript attached. The immunoassay method has been submitted to Analytical Biochemistry.
- We synthesized peptide-nanoparticles conjugates with different valencies (numbers of peptides per nanoparticle) and evaluated them for their interaction with PC-3 cells. Maximal performance was achieved when there are about 30 peptides using an arginine series of linker amino acids to the nanoparticle.
- W evaluated peptide-nanoparticles for their growth promoting properties on PC-3 cells. We observed that peptide-nanoparticle conjugates were growth inhibitory while bombesin like peptides had, as expected, a growth promoting activity.

Conclusions:

The FITC-hapten immunoassay method is an accurate method and can be used to assess the interaction of peptides or peptide-nanoparticles with cells.

Bombesin-like peptides with a spacer of arginine provide the strongest interaction with PC-3 cells, stronger than spacers of neutral or negatively charged amino acids.

The optimum valency for the design of peptide-nanoparticle conjugates is about 30 peptides per nanoparticle.

Bombesin-like peptides, when attached to nanoparticles, loose their growth promoting properties.

Reportable Outcomes:

The FITC-hapten immunoassay method is an accurate method and can be used to assess the interaction of peptides or peptide-nanoparticles with cells. We expect our method to be published.

We have optimized nanoparticle design for use targeting the gastrin releasing peptide receptor expressed on PC-3 cells.

Title: FITC-hapten immunoassay for determination of peptide-cell interactions

Authors: Kimberly A. Kelly, Fred Reynolds, Ralph Weissleder and Lee Josephson*

All authors are from: Center for Molecular Imaging Research, Massachusetts General Hospital, Charlestown, MA 02129, USA

*Corresponding author

Lee Josephson

Phone: 617-726-6478

FAX: 617-726-5708

Email: ljosephson@partners.org

Abstract

We have developed a FITC-hapten immunoassay, where a FITC-labeled peptide binding to a cell is assayed as the amount of immunoreactive fluorescein present in a cell lysate. An anti-fluorescein/horseradish peroxidase conjugate binds to either a fluoresceinated peptide in the lysate or with a fluorescein attached to the wells of a microtiter plate in a competitive fashion. After washing, solid phase peroxidase activity is measured and inversely related to the amount of FITC-labeled peptide present. To demonstrate the assay, the interaction of a FITC-labeled bombesin-like peptide with the gastrin releasing peptide receptor on PC-3 cells and HT-29 cells was investigated. Using PC-3 cells, we obtained similar displacement curves and numbers of binding sites per cell by both FITC-hapten immunoassay and a reference radioreceptor assay. The FITC-hapten immunoassay is a sensitive and versatile method, since the same commercially available reagents can be used to assess interactions between any peptide and any receptor. In addition, the FITC-labeled peptide can be used to visualize receptors in fluorescent activated cell sorting or by fluorescent microscopy.

Abbreviations: FITC, fluorescein isothiocyanate; DTT, dithiothreitol; ANS, 8-anilino-1-naphthalenesulfonic acid; PBS, phosphate buffered saline; FBS, fetal bovine serum; HRP, horseradish peroxidase; BSA, bovine serum albumin; TMB, tetramethyl benzidine.

Introduction

While techniques for assaying the interactions of ligands with purified receptors are common, cell-based receptor assays are invaluable in cases where the cell has an unidentified receptor, where the receptor is difficult to purify, or where binding is a function of interactions between the receptor and regulatory cellular proteins. The quantitation of cell-associated peptide in cell-based receptor assays is often obtained by determining levels of cell-associated radioactivity. Typically peptides are synthesized and labeled with ^{125}I monthly, to overcome radiochemical decay. In addition to the usual issues of laboratory contamination, disposal costs, and frequent synthesis, iodinated tracers undergo a de-iodination reaction which can occur either with storage or in vivo [1-3].

To remedy these difficulties, we have developed a FITC-hapten immunoassay, where the fluorescein of fluoresceinated peptides serves as a tracer in a competitive format enzyme immunoassay. An anti-fluorescein horseradish peroxidase conjugate (anti-FITC-HRP) reacts with a FITC containing peptide in a cell lysate and then unreacted conjugate reacts with a solid phase fluorescein coated on the well of a microtiter plate. After washing, solid phase enzyme

activity is determined. The use of FITC as a hapten for immunological detection has many advantages. In addition to being highly stable, the FITC-labeled peptides can be used as probes in fluorescence based methods such as fluorescent activated cell sorting or by fluorescence microscopy. Second, the conjugation of FITC to peptides can be achieved during automated solid phase peptide syntheses, further minimizing the labor. Third, the FITC-hapten immunoassay permits a single set of reagents, the anti-FITC-HRP conjugate and a solid phase fluorescein, to be used to assay for any FITC-labeled peptide.

To demonstrate the utility of the FITC-hapten immunoassay, we determined the interactions of bombesin with the well-characterized gastrin releasing peptide receptor (GRP receptor), an important growth promoting receptor found on a variety of cell types [4-6] We show that the FITC-hapten immunoassay is a versatile, non-isotopic method of determining the interaction between peptides and receptors expressed on cells.

Materials and Methods

Peptide synthesis: All peptides were synthesized on an Advanced Chemtech Apex 396 peptide synthesizer using standard FMOC chemistry and Rink amide resin. All peptides were prepared as C-terminal amides. The Dde group was removed with 10 mL of 2% hydrazine in DMF (2 X 3 min) and the deprotected amino group was reacted with 0.4 mmoles of FITC (0.1 mmole solid phase peptide) in 5 mL of DMSO/diisopropylethylamine (20% v/v) overnight. Peptide was then cleaved from the solid support (5 mL of TFA/thioanisole/ethandithiol/anisole (90/5/3/2)) and purified by C18 reversed-phase HPLC. The identity of peptides was confirmed by MALDI-TOF and was within 1 dalton of their expected molecular weight in all cases. Peptides used were K(FITC)D, GRKKRRQRRRGYK(FITC)C or tat(FITC), GRRRRRGRRRRGyK(FITC) or polyarg(FITC), and the bombesin-like peptide CK(FITC)RRRQWAVGHLM, denoted BN(FITC). All peptides were C-terminal amides.

FITC-Hapten Assay: The FITC-hapten immunoassay is a competitive type enzyme immunoassay where sample is first incubated with an anti-FITC-HRP conjugate, and then added to a FITC coated microtiter plate (scheme shown in Figure 1A). In the absence of fluorescein in the sample, anti-HRP binds

to the FITC coated plate and, after washing, a high level of HRP enzyme activity is obtained. In the presence of fluorescein the anti-FITC-HRP fails to bind to the microtiter plate.

The standard assay protocol used 96 well microtiter plates (Nalgene Maxi-sorp) coated with FITC-BSA (Sigma Chemical) at a FITC concentration of 0.8 nM FITC or 12.5 ng/mL BSA (300 uL/well). Coating was in PBS, pH 7.4 overnight at 4°C. The following day, plates were washed three times with blocking buffer (PBS pH 7.4, 0.1% BSA, and 0.1% Tween-20) and incubated (30-60 minutes) with blocking buffer at room temperature followed by an additional wash in blocking buffer containing 2% glucose. Plates were allowed to air dry then stored at 4 °C.

Standards or unknowns were prepared by diluting the K(FITC)D peptide (standard) or unknown in one of two buffers (PBS pH 7.4, 0.1% BSA, 1mM ANS or PBS pH 7.4, 0.1% BSA, 1mM ANS, 1% Triton X-100), which gave equivalent results as shown Figure 3B. Five uL of anti-FITC-HRP (Molecular Probes) diluted to 4 ug/mL in blocking buffer was added per 500 uL of sample or standard to give a final concentration of 40 ng/mL conjugate. The conjugate was allowed to bind FITC in the

sample for 2 hours at room temperature. Some 200 uL was then transferred to a well of the FITC-BSA coated microtiter plate. Anti-FITC-HRP was allowed to bind the solid phase FITC for an additional hour at room temperature. Following incubation, plates were washed 3 times with blocking buffer containing 0.1% Tween-20, to remove unbound antibody, and 200 uL TMB substrate (Sigma Chemical) was added. Absorbance at 650 nm was obtained after 30 minutes at room temperature on a microtiter plate reader (Gemini, Molecular Devices).

Standard curves for FITC were obtained by plotting normalized absorbance in percent against the logarithm of analyte concentrations. Normalized absorbance is the absorbance divided by maximum absorbance, or absorbance obtained using a standard containing no fluorescein, and multiplying by 100. Data were fit to the basic four-parameter logistic equation where: Y=m1+(-m2-m1)/(1+10^(m3-m0)*m4). Here m0 is the hapten concentration, m1 is the maximum absorbance value, m2 is the curve slope at the inflection point, m3 is the concentration of hapten that gives 50% inhibition, and m4 is the minimum absorbance.

Determination of anti-FITC/HRP affinity for FITC: Microtiter plates were coated with 0.08 nM of FITC conjugated to BSA as

above (FITC-BSA). After plates were washed and blocked, increasing concentrations of anti-FITC/HRP were incubated with the plate for 1 hour. Following incubation, plates were washed, substrate was added, and the absorbance at 650 nm determined. The bound and total concentrations of anti-FITC-HRP were obtained using a standard curve (enzyme activity of anti-FITC-HRP dilutions versus absorbance at 650 nm) and analyzed by Scatchard analysis.

Cell culture: PC-3 and HT-29 cell lines were obtained from ATCC. Cells were grown at 37 °C with 5% CO2. PC-3 cells were grown in F12K medium supplemented with 10% fetal bovine serum and penicillin/streptomycin. HT 29 cells were cultured in Dulbecco's Modified Medium supplemented with 4 mM glutamine, 10% fetal bovine serum, and pen/strep. Cells were passaged by the addition of trypsin when confluent.

Radioreceptor and FITC-hapten receptor assays: We used 24 well plates with PC-3 cells at a cell density of $1x10^5$ cells/well and HT-29 cells at a cell density of $2.5x10^5$ cells/well. Cells were trypsinized, plated, and used the following day. Two displacement assays were performed in order to compare the kinetics of competition obtained using radiation or the FITC immunoassay. For the determination of

the IC_{50} of bombesin by radioreceptor assay, ^{125}I-labeled [Tyr^4] bombesin (Perkin-Elmer Bioscience) at a final concentration of 3 fM was mixed with increasing concentrations of unlabeled bombesin peptide and added to the culture medium on the cells. Cells were incubated for 2 hrs at 37°C, washed twice with HBSS, and lysed with PBS/0.1%BSA/1mM ANS/0.1% Triton X-100. Radioactivity in the lysate was determined (PE Wizard 3 gamma counter). To determine the IC_{50} of competition between FITC-bombesin and bombesin, increasing log concentrations of BN(FITC) were added to a constant concentration of bombesin and incubated with PC-3 cells for 2 hours at 37°C. Following incubation, cells were washed twice with HBSS and lysed using PBS/0.1% BSA/1mM ANS/0.1% Triton X-100. FITC was quantitated using the optimized FITC immunoassay conditions. For the FITC-bombesin uptake assays, increasing concentrations of BN(FITC) were incubated with PC-3 or HT-29 cells for 2 hours at 37°C. Following incubation, cells were washed then lysed with PBS/0.1%BSA/1mM ANS/0.1% Triton X-100 and the amount of FITC bound to the cells quantitated with the FITC immunoassay.

Results and Discussion

Assay optimization and characterization: To optimize the assay, we varied concentration of FITC-BSA used for coating and the concentration of anti-FITC-HRP as shown in Figure 1B. We selected 40 ng/mL of anti-FITC-HRP and 8 ng/mL of BSA as the standard conditions. A standard curve is shown in Figure 1C using K(FITC)D as a standard. To obtain assay sensitivity, the lowest value statistically different from zero, the standard deviation at the zero FITC concentration was obtained (5.6%, n= 40) as normalized absorbance and subtracted the maximum absorbance (94.4%). This corresponded to a concentration of 78 fM using the standard curve (Figure 1C) or to the detection of 1.6×10^{-17} moles of FITC.

We next characterized the affinity of the anti-FITC-HRP for solid phase FITC as shown in Figure 2. Data were analyzed by the method of Scatchard to give a Kd of 637 pM, with 7.8 x 10^8 binding sites per well, see inset.

A series of chemically different fluoresceinated peptides were employed as standards, to determine if the nature of the amino acids surrounding the fluoresceinated epsilon amino group of lysine affected the interaction between fluorescein and anti-FITC-HRP (Figure 3A). K(FITC)D is a

short hydrophilic anionic peptide, while tat(FITC) and PolyR(FITC) are cationic peptides. The BN(FITC) peptide is a zwitter ionic and hydrophobic peptide. As shown in Figure 3A, peptides with widely differing sequences were detected similarly by the assay. We next evaluated the effects of assay media and obtained similar standard in media with or without 10% fetal calf serum (Figure 3B). The FITC-hapten immunoassay is highly specific and insensitive to biological fluids like serum, which was expected because molecules similar to fluorescein are not found in biological systems. The specificity of the assay (crossreactvity) with different fluorochromes (eosin, rhodamine and Cy5.5) was found to be less than 1%. Interestingly, photobleaching fluorescein had no effect on its immunoreactivity, presumably because antibody is insensitive to subtle electronic rearrangements.

Determination of peptide-receptor interactions in cell-based assays: A standard method of assessing the interactions of peptides with receptors is to determine the binding of iodinated peptides to receptors expressed on cultured cells. We determined the binding of bombesin to the GRP receptor on PC-3 cells using displacement by bombesin in both radioreceptor (^{125}I-[Tyr^4] bombesin tracer) and the FITC

hapten (BN(FITC) tracer) immunoassay methods as shown in Figure 4. PC-3 cells are a human prostate cancer cell line that expresses the GRP receptor [6]. As shown in Table 1, both methods yielded similar IC_{50}'s and numbers of sites per cell [6]. As expected, the HT-29 cells, which lack the GRP receptor, had a low number of binding sites per cell [7]. Using radioactive bombesin, similar IC_{50}'s for the GRP receptor have been obtained [8, 9].

The use of the FITC/anti-FITC antibody system rather than biotin/avidin for our assay has several advantages. First, FITC based reagents can be used with fluorescence-based modalities like FACS or fluorescent microscopy. In addition, FITC provides a structure that is not found in nature and antibodies are very unlikely to cross react with unknown but naturally occurring analytes. Though avidin binds biotin tightly, biotinylated peptides could not be employed because biotin is a naturally occurring substance and could be present in cell lysates [10]. Finally, a single set of commercially available reagents can be used to assay for any cell-associated FITC-labeled peptide, FITC-labeled protein, or for FITC-labeled magnetic nanoparticles (Josephson, unpublished observations).

Conclusions

We present a microtiter plate based, competitive format enzyme immunoassay to quantitate the hapten FITC attached to peptides. The FITC hapten immunoassay is used to measure the amount of FITC-labeled peptides interacting with receptors on cells in culture in a manner similar to radioreceptor assays. FITC hapten immunoassay is sensitive, specific, free from all known interferences and provides an attractive alternative to radioactive peptide binding assays in cell-based systems.

References

(1) Colin, S.; Mascarelli, F.; Jeanny, J. C.; Vienet, R.; Bouche, G.; Courtois, Y.; Labarre, J. *Eur J Biochem* **1997**, *249*, 473-480.

(2) Baumann, G.; Amburn, K. *J Immunoassay* **1986**, 7, 139-149.

(3) Lee, D. S.; Griffiths, B. W. *J Immunol Methods* **1984**, *74*, 181-189.

(4) Zachary, I.; Rozengurt, E. *Proc Natl Acad Sci U S A* **1985**, *82*, 7616-7620.

(5) Cuttitta, F.; Carney, D. N.; Mulshine, J.; Moody, T. W.; Fedorko, J.; Fischler, A.; Minna, J. D. *Nature* **1985**, *316*, 823-826.

(6) Reile, H.; Armatis, P. E.; Schally, A. V. *Prostate* **1994**, *25*, 29-38.

(7) Radulovic, S. S.; Milovanovic, S. R.; Cai, R. Z.; Schally, A. V. *Proc Soc Exp Biol Med* **1992**, *200*, 394-401.

(8) Frucht, H.; Gazdar, A. F.; Park, J. A.; Oie, H.; Jensen, R. T. *Cancer Res* **1992**, *52*, 1114-1122.

(9) Mantey, S.; Frucht, H.; Coy, D. H.; Jensen, R. T. *Mol Pharmacol* **1993**, *43*, 762-774.

(10) Mock, D. M. *Methods Enzymol* **1997**, *279*, 265-275.

Acknowledgment:

This work was supported in part by US Army grant DAMD 170210089.

Table 1: FITC-hapten and radioreceptor assays for bombesin binding

Cells	Assay	IC_{50} (nm)	Sites/Cell
PC-3	FITC-hapten	8.7	14,000
PC-3	Radio receptor	6.3	13,000
HT-29	FITC-hapten		<1000

Figure Legends

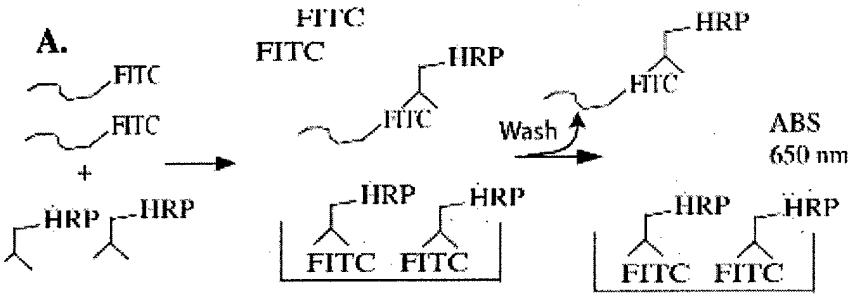
Figure 1: FITC-hapten immunoassay. (A) Scheme of FITC immunoassay procedure. FITC-labeled peptide is incubated with anti-FITC/HRP antibody and the mixture transferred to a FITC coated microtiter plate. Unbound (free) antibody is removed and the presence of the antibody is detected as HRP. (B) Assay optimization (-) absorbance <0.25; (+) absorbance 0.25-0.5; (++) absorbance 0.5-0.75; (+++) >0.75. (B) typical standard FITC curve for optimized assay (8 nM FITC, 40 ng/mL anti-FITC-HRP).

Figure 2: Characterization of the Anti-FITC/HRP antibody binding FITC-BSA. Inset shows Scatchard analysis.

Figure 3: FITC-hapten immunoassay characterization (A): Standard curves with fluoresceinated peptides of different sequences. Peptides were: ■, BN(FITC); ▲, polyarg(FITC); ●, K(FITC)D; ◆ tat(FITC). B) Standard curves with the peptide K(FITC)D as a standard in different assay media. Media were: ▲, 10% serum ; ●, PBS-BSA; ◆ 1.0% Triton X-100.

Figure 4: Binding to PC-3 cells determined by FITC-hapten immunoassay and radioreceptor assay. ■, % absorbance; ●, radioactivity.

Figure 1:



B.

AntiFITC-HRP (ng/mL)	FITC-BSA (nM, FITC) 0.8	8.0
4	-	+
40	++	+++
400	+++	+++

C.

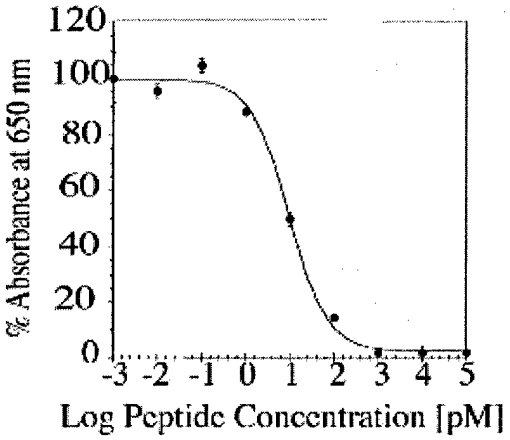


Figure 2:

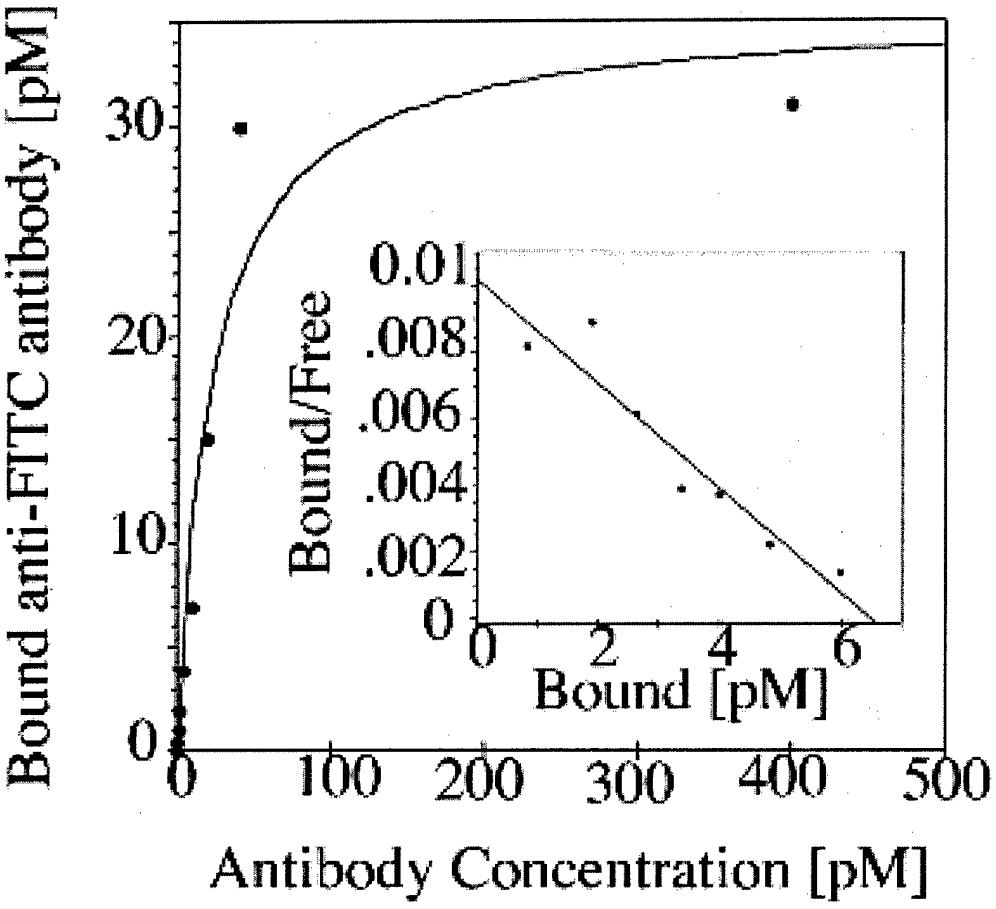

Bound anti-FITC antibody [pM]
30
20
10
0
0
100
200
300
400
500
Antibody Concentration [pM]
Bound/Free
0.01
.008
.006
.004
.002
0
0
2
4
6
Bound [pM]

Figure 3

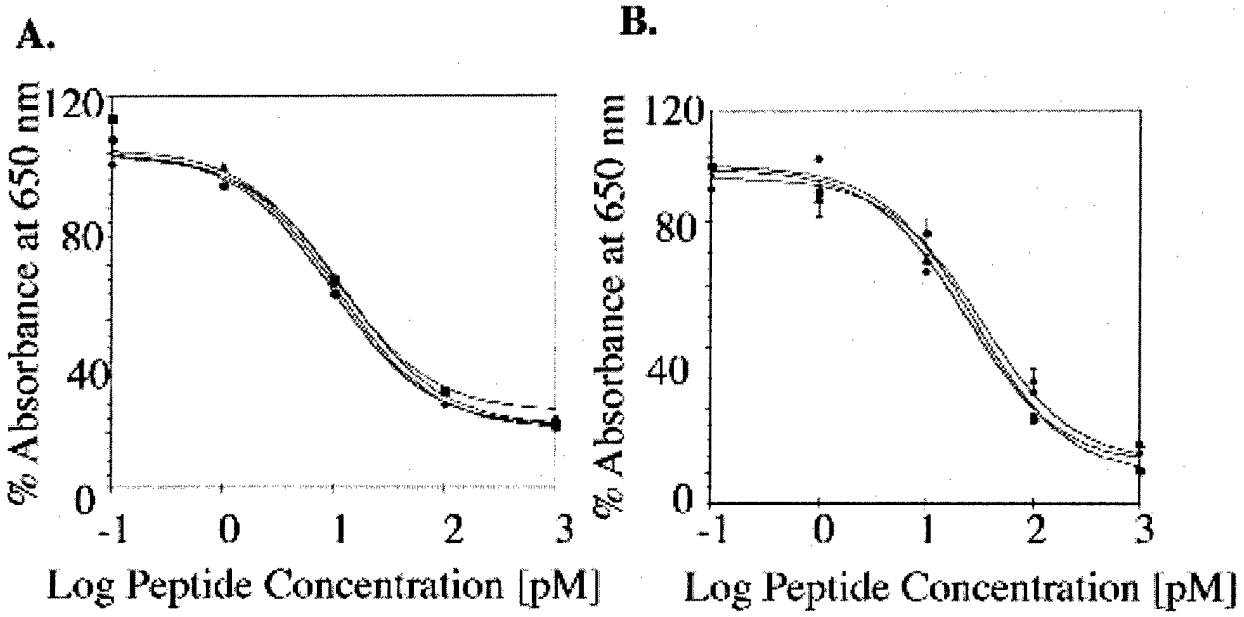
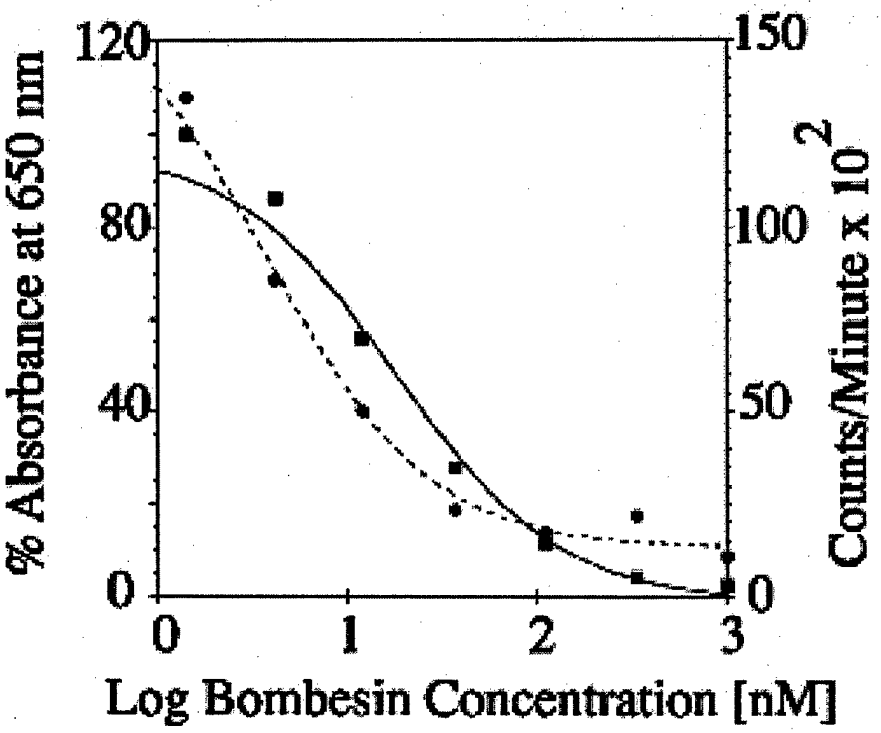

A.
B.
% Absorbance at 650 nm
120
80
40
0
-1
0
1
2
3
Log Peptide Concentration [pM]

Figure 4:


120
80
40
0
% Absorbance at 650 nm
150
100
50
0
Counts/Minute x 10 2
0
1
2
3
Log Bombesin Concentration [nM]